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Title:

Kinetic analysis of the transphosphorylation with creatine kinase by pressure-assisted capillary electrophoresis/dynamic frontal analysis

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Abstract:

Kinetic reactions of the transphosphorylation with creatine kinase (CK) were individually investigated between creatine (Cr) and creatine phosphate (CrP) by pressure-assisted capillary electrophoresis/dynamic frontal analysis (pCE/DFA). The transphosphorylations are reversible between Cr and CrP, and reverse reactions inevitably accompany in general batch analyses. In pCE/DFA, the kinetic reaction proceeds in a separation capillary and the product is continuously resolved from the substrate zone. Therefore, the formation rate is kept constant at the substrate zone without the reverse reaction, and the product is detected as a plateau signal. This study demonstrates the direct and individual analyses of both the forward and the backward kinetic reactions with CK by pCE/DFA. A plateau signal was detected in the pCE/DFA with ADP or ATP as one of the products on either the forward or the backward reactions. The Michaelis-Menten constants of $K_{m,ATP}$ (from Cr to CrP) and $K_{m,ADP}$ (from CrP to Cr) were successfully determined through the plateau signal. Determined values of $K_{m,ATP}$ and $K_{m,ADP}$ by pCE/DFA were smaller than the ones obtained by the pre-capillary batch analyses. The results agree with the fact that the reverse reaction is excluded in the analysis of the kinetic reactions. The proposed pCE/DFA is useful on individual analyses of both forward and backward kinetic reactions without any interference from the reverse reaction.

Keywords: Capillary electrophoresis, dynamic frontal analysis, creatine kinase, transphosphorylation, kinetic analysis

Declarations:

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Code availability: **N/A**

Authors' contributions: **M. M.**: Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing - original draft; **H. M.**: Investigation, Resources, Validation; **T. T.**: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing - review & editing.

Introduction

Enzymatic reaction with transferase directs the selective transfer of a substituent from one substrate to another substrate [1]. Transphosphorylation with a kinase (phosphotransferase) is one of the popular transfer reactions *in vivo*. A specific substrate receives a phosphate group from an adenosine triphosphate (ATP), and the ATP itself is converted to an adenosine diphosphate (ADP) in the transphosphorylation. The transphosphorylation is reversible, and an ADP also receives a phosphate group by the transphosphorylation and it becomes an ATP. In this way, ATP and ADP are common substrates in the transphosphorylation for various kinases such as adenosine kinase [2], hexokinase [3], tyrosine kinase [4], sphingosine kinase [5], creatine kinase [6], and protein kinase [7-10]. Creatine kinase (CK, EC 2.7.3.2, also called as creatine phosphokinase) is an enzyme that is widely found in muscle tissues and brains [11,12]. CK catalyzes the reversible transfer of a phosphate group from ATP to creatine (Cr), as well as from creatine phosphate (CrP) to ADP [11]. The reversible reaction is expressed as in reaction (1).

$$\mathrm{Cr} + \mathrm{ATP} \underset{\mathrm{CK}}{\rightleftharpoons} \mathrm{CrP} + \mathrm{ADP} \qquad (1)$$

The transphosphorylation with CK plays an important role in energy conservation by transferring a phosphate group from CrP to ADP, when the amount of ATP is reduced by sugar metabolisms [13,14]. An elevated level of CK is an important indicator of the muscle damage in clinical examinations, and the measurement of CK activity in blood is used as a diagnostic test for possible muscle diseases [15].

Capillary electrophoresis (CE) is a powerful analysis tool for ionic species, and the separation criteria have widely been used for enzyme assays [16]. Enzymatic reactions are sometimes done in a separation capillary, and in-capillary analyses of enzyme assays are popularly called as: electrophoretically mediated microanalysis (EMMA) [17-20], immobilized enzyme reactor (IMER) [21-23], pressure mediated microanalysis (PMMA) [24], or transverse diffusion of laminar flow profiles (TDLFP) [25,26]. When the enzymatic reactions are done by in-capillary reactions, box-shaped signals are sometimes detected by the continuous mode in the EMMA format [27,28]. However, most EMMAs are simply the coupling of a batch reaction in a capillary with CE separation and quantification of the products. As for the CE analyses, the CK activity was measured by pre-capillary [29,30] and in-capillary reactions [30], but the Michaelis-Menten analysis was not successful. Because the CE analyses are based on batch reactions. In batch reactions whether it is pre-capillary or in-capillary reaction, the amount of the product increases along with the progress in the forward reaction, and the backward reaction, a reverse reaction, subsequently proceeds and inhibits the forward reaction. As far as the reaction product is involved, the individual analyses of the forward or backward reaction of CK are difficult.

The present authors have recently developed an enzyme assay of capillary electrophoresis/dynamic frontal analysis (CE/DFA) as a technique to monitor the steady-state of an enzymatic reaction in a capillary [31-33]. In CE/DFA,

a zero-order kinetic reaction continuously proceeds at the substrate zone, and the product is immediately resolved from the substrate zone by the electrophoresis. A plateau signal can be detected as a result of the steady-state of the kinetic reaction, and the plateau height can directly be used for the analysis of the zero-order kinetic reaction [31-33]. Since the product is continuously resolved from the substrate zone, the enzyme inhibition by the product can be eliminated by CE/DFA, as well as one of EMMA formats [18].

In this study, the characteristic of CE/DFA, immediate resolution of the product from the substrate zone, has been utilized for the individual analyses of both the forward and the backward kinetic reactions of CK. Pressure-assist was also utilized in CE/DFA for the fast detection of the plateau signal as reported with carboxylesterase [34]. Both the forward and the backward reactions with CK have individually analyzed by the pressure-assisted capillary electrophoresis/dynamic frontal analysis (pCE/DFA), and the corresponding Michaelis-Menten constants have successfully been determined through the plateau signals without any interference from the product.

Reaction scheme with creatine kinase as reversible kinetic reactions in pCE/DFA

The laminar flow profile is generated by the pressure assist, and the laminar flow profile develops the boundary area between adjacent zones [25]. Mixing of the zones is also promoted by the laminar flow. TDLFP is widely used for the analysis of kinases and it is useful for rapid analyses [35-38]. The characteristics of the pressure assist were utilized in this study as pCE/DFA.

In this study, the forward and the backward reactions with CK were individually analyzed by pCE/DFA. Figure 1 schematically illustrates the stepwise stages of the enzymatic reaction in pCE/DFA. CrP and ADP are generated from Cr and ATP in the forward reaction; thus, the separation capillary is filled with Cr and CK, where an ATP solution is introduced into the capillary as a sample plug (Fig. 1, F1). When a DC voltage and air pressure are applied to the capillary, the ATP zone electrophoretically migrates in the separation buffer and the enzymatic reaction proceeds during the migration of the substrate ATP in the capillary. Products of CrP and ADP are continuously generated in the ATP zone, and they are immediately resolved from the substrate zone (Fig. 1, F2). pCE/DFA is different from the conventional CE/DFA and mixing of the zones is accompanied in the pCE/DFA based on the parabolic flow. When the detection wavelength is set at 260 nm, only ADP can be detected among the products, as well as the substrate ATP. At the pH condition of 7.4, the ATP migrates slower than the ADP during the pCE due to its higher negative charge. As a result, continuously generated and resolved ADP would be detected as a plateau signal and the substrate ATP zone would follow as a peak signal (Fig. 1, F3). The plateau height of ADP is subjected to the kinetic analysis. In the analysis of the backward reaction, the capillary is filled with CrP and CK and a sample solution containing ADP is injected into the capillary (Fig. 1, B1). The enzymatic reaction also proceeds by the migration of ADP in the capillary, and ATP and Cr are continuously generated and resolved (Fig. 1, B2). Based on the electrophoretic mobility of ATP and ADP, the ADP zone would be detected as a peak signal

and the ATP would follow as a plateau signal (Fig. 1, B3). The plateau height of ATP is also subjected to the kinetic analysis.

Fig. 1

Materials and methods

Materials and reagents

Creatine kinase (CK; from rabbit muscle, EC: 2.7.3.2) and adenosine 5'-triphosphate (ATP, received as disodium salt) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Adenosine 5'-diphosphate (ADP, received as disodium salt), creatine (Cr), and creatine phosphate (CrP) were from Fujifilm Wako Pure Chemical (Osaka, Japan). All other reagents were of analytical grade. Water used was purified by a Milli-Q Gradient A10 (Merck-Millipore, Milford, MA, USA).

Apparatus

The CE apparatus and the fused-silica capillary attached to the CE system are essentially the same as in our previous studies [33,34]. The capillary cartridge was thermostated at 30 °C by circulating constant temperature air. In the pre-capillary reactions, the reaction was done at the vial tray of the system, and the vial tray was also thermostated at 30 °C by circulating temperature-controlled water. The capillary was conditioned daily in an ordinary manner, and it was equilibrated with each separation buffer by filling it and holding on for 1 h before the pCE/DFA. The control of the CE system, the data acquisition, and the data analysis are the same as in our previous studies [33,34].

Procedure for the CE analyses

Pre-capillary reaction for the determination of the Michaelis-Menten constants

On the analysis of the forward reaction forming CrP and ADP from Cr and ATP as written in reaction (1), an enzymatic reaction was made in an aliquot of 750 μL solution containing varying concentrations of ATP, 8.0 mmol L^{-1} Cr, 4.0 u/mL CK, and 10 mmol L^{-1} phosphate buffer (pH 7.4). The solution was incubated for 8 min at 30 °C at the vial tray of the CE system. The incubated solution was hydrodynamically injected into the separation capillary at 50 mbar for 3 s from the anodic end. After each ends of the capillary were dipped in the separation buffer vials containing 10 mmol L^{-1} phosphate buffer (pH 7.4), a DC voltage of 20 kV was applied to the capillary and an air pressure of 20 mbar was applied to the inlet vial for the pCE. Products of CrP and ADP were photometrically detected at 200 nm. All CE measurements for the pre-capillary reactions were made three times for the reproducibility verification.

On the analysis of the backward reaction forming Cr and ATP from CrP and ADP as written in reaction (1), an enzymatic reaction was similarly made in an aliquot of 750 μL solution containing varying concentrations of ADP, 2.0 mmol L^{-1} CrP, 4.0 u/mL CK, and 10 mmol L^{-1} phosphate buffer (pH 7.4). The solution was incubated for 5 min at 30 °C at the vial tray of the CE system. After the incubated solution was hydrodynamically injected into the separation capillary at 50 mbar for 3 s, a DC voltage of 20 kV was applied to the capillary for the CE. The product of Cr was photometrically detected at 200 nm.

Inhibition of a product ATP in the backward reaction was also examined in the pre-capillary reaction. Different concentrations of ATP were added in the reaction vial containing 1.0 mmol L^{-1} ADP, 2.0 mmol L^{-1} CrP, 4.0 u/mL CK, and 10 mmol L^{-1} phosphate buffer (pH 7.4). The solution was incubated for 5 min at 30 °C at the vial tray of the CE system. A DC voltage of 20 kV was applied to the capillary for the CE. The product of Cr was detected at 200 nm.

Individual measurements of the forward and the backward reactions by pCE/DFA

On the analysis of the forward reaction forming ADP from ATP as written in reaction (1), a solution containing 0.3-2.0 mmol L^{-1} ATP and 10 mmol L^{-1} phosphate buffer (pH 7.4) was used as a sample solution. A solution containing 4.0 u/mL CK, 16 mmol L^{-1} Cr, and 10 mmol L^{-1} phosphate buffer was used as a separation buffer. After the separation capillary equilibrated with the separation buffer, the substrate solution was hydrodynamically injected into the capillary from the anodic end of the capillary by applying a pressure at 50 mbar for 3 s. After each ends of the capillary were dipped in the separation buffer vials, a DC voltage of 20 kV was applied to the capillary and an air pressure of 20 mbar was applied to the inlet vial for the pCE. A substrate ATP and the product ADP were photometrically detected at 260 nm. Cr and CrP did not interfered with the photometric detection of ATP and ADP at this wavelength. All pCE measurements were made three times for the reproducibility verification.

In the backward reaction forming ATP from ADP, a solution containing 0.5-3.0 mmol L^{-1} ADP and 10 mmol L^{-1} phosphate buffer (pH 7.4) was used as the sample solution. A solution containing 2.0 u/mL CK and 8.0 mmol L^{-1} CrP, 10 mmol L^{-1} phosphate buffer (pH 7.4) was used as the separation buffer. The pCE conditions are the same as in the forward reaction.

Determination of the Michaelis-Menten constants

Both the forward and the backward reactions are enzymatic reaction, and each of them possesses Michaelis-Menten constant. The Michaelis-Menten constant of the forward reaction is conventionally defined as $K_{m,ATP}$, as written in the reaction (1a). The Michaelis-Menten constant of the backward reaction is similarly defined as $K_{m,ADP}$, as written in the reaction (1b).

$$\mathrm{Cr} + \mathrm{ATP} \xrightarrow[\mathrm{CK}]{K_{\mathrm{m,ATP}}} \mathrm{CrP} + \mathrm{ADP} \quad (1a)$$

$$\mathrm{CrP} + \mathrm{ADP} \xrightarrow[\mathrm{CK}]{K_{\mathrm{m,ADP}}} \mathrm{Cr} + \mathrm{ATP} \quad (1b)$$

Michaelis-Menten kinetic analysis was made with a non-linear least-squares analysis of the Michaelis-Menten curve represented by equation (2). A Michaelis-Menten constant (K_m) can be determined with several data sets of the substrate concentration, [S], and the reaction rate, v. An R program (Ver 4.0.3) was used for the non-linear least-squares analysis [39]. In this study, the plateau height was used instead of the reaction rate, since the plateau height is directly related with the reaction rate [31,32].

$$v = \frac{V_{\max}[\mathrm{S}]}{K_{\mathrm{m}} + [\mathrm{S}]} \quad (2)$$

Results and Discussion

Determination of the Michaelis-Menten constants by the pre-capillary reaction

In the forward kinetic reaction, Michaelis-Menten analysis was made in a series of ATP concentrations ranging from 0.3 to 2.0 mmol L^{-1}. CrP and ADP were generated from Cr and ATP by the transphosphorylation with CK; typical electropherogram by the pre-capillary reaction is shown in Fig. 2A. Since the electric charges of ADP, ATP, and CrP are highly negative, it took long migration time. Therefore, pCE was used for the fast detection of the substances. It is noted from Fig. 2A that both ADP and CrP are formed from ATP and Cr. A peak of CrP was broadened by the pressure assist, and the peak area of ADP was used for the Michaelis-Menten analysis.

The backward kinetic reaction was also examined with the ADP concentrations ranging from 0.5 to 3.0 mmol L^{-1}. Since acid dissociation constants of Cr are pK_{a1} = 2.63 (from monocation to zwitterion) and pK_{a2} = 14.30 (from zwitterion to monoanion) [40], the charge of Cr is almost zero at the pH conditions examined. And therefore, one of the products, Cr, can be detected at the migration time of the electroosmotic flow, and the pressure assist was not used in the CE measurements. A typical electropherogram by the pre-capillary reaction is shown in Fig. 2B; a product Cr is detected. The peak area of Cr was used for the Michaelis-Menten analysis.

Non-linear least-squares analyses have been made with the peak area; the analysis plots are shown in Fig. 3. Michaelis-Menten constants of $K_{m,ATP}$ = 0.58±0.03 mmol L^{-1} and $K_{m,ADP}$ = 1.91±0.16 mmol L^{-1} were obtained. Fitted curves are drawn with the analysis results. Both K_m values obtained in this study are close to the reported values [41-44].

The results are summarized in Table 1.

Fig. 2

Fig. 3

Table 1

Inhibition with ATP in the backward reaction by the pre-capillary reaction

Since the transphosphorylation is a reversible enzymatic reaction, the forward or the backward reaction can be inhibited with the product ATP or ATP by its reverse reaction. The inhibition effect of ATP in the backward reaction (1b) was investigated by the pre-capillary reaction. A series of an adequate amount of ATP were added to the sample solution as an inhibitor, and the inhibition was monitored through the decrease in the peak area of Cr. The peak area of Cr obtained without ATP in the initial sample solution was set as 100 % enzyme activity. The decrease in the enzyme activity is shown in Fig. 4. It can be noted from Fig. 4 that the enzyme activity in the backward reaction decreased with increasing concentrations of ATP; IC_{50} = 0.70 mmol L^{-1} was obtained. Unlike general enzymatic hydrolysis reactions, reversible reactions such as transphosphorylation are suppressed by the backward reaction. Since this CE analysis monitored the Cr concentration in the pre-capillary reaction, one of the products of Cr was not added in the sample solution. If Cr was supplementary added in the sample solution, the product inhibition would be expected even greater.

Fig. 4

Determination of the Michaelis-Menten constants by pCE/DFA

The forward and the backward enzymatic reactions were examined in the pCE/DFA format, as illustrated in Fig. 1. The results are shown in Fig. 5A and 5B, respectively. When an ATP solution was introduced into the capillary as a sample plug, an ADP plateau was detected by the enzymatic transphosphorylation in the pCE/DFA format (Fig. 5A). A flat plateau is detected in each electropherogram. The flat profile suggested that the enzymatic transphosphorylation proceeded at a constant reaction rate, or the transphosphorylation is at a steady-state. This is because the product ADP is continuously resolved from the ATP zone and the inhibition with the product or the reverse reaction is excluded. Therefore, the pCE/DFA worked well in this enzymatic phosphorylation. The plateau height increased with increasing concentrations of ATP in the sample solution, as in (a) to (c). Harmon, *et al.* examined the reversible oxidation with alcohol dehydrogenase by an in-capillary EMMA format, and the electrophoretic resolution of the product from the substrate zone was utilized for quantifications of the substrate and the enzyme; the product NADH was quantified through the integration of the absorbance response of the product, because the enzymatic reaction was not under the steady-state and any plateau

signal was not obtained [18].

Typical electropherograms of the backward reaction are also shown in Fig. 5B, where an ADP solution was introduced into the capillary as a sample plug. A plateau signal of the product ATP is also detected in each electropherogram. The plateau signal gradually increased with the migration time in Fig. 5B (d). This result is attributed to the insufficient concentration of the substrate ADP. The substrate ADP is rather sufficient and the reaction rate is high at the start of the kinetic reaction (late migration time). Along with the progress of the enzymatic reaction, the substrate ADP is consumed and the reaction rate is gradually reduced (early migration time). The shortage of ADP was developed at higher concentrations of ADP, as in Fig. 5B (f); a flat plateau was obtained. For the declined plateau signals as Fig. 5B (d), the highest point of the plateau (initial reaction rate) was used for the kinetic analysis of the enzymatic reaction.

Fig. 5

Figure 6 shows non-linear least-squares analyses of the forward and the backward reactions. Michaelis-Menten curves were obtained with each reaction. Michaelis-Menten constants were determined as $K_{m,ATP}$ = 0.39±0.03 mmol L^{-1} and $K_{m,ADP}$ = 1.08±0.10 mmol L^{-1} by the analysis. Both values are smaller than the ones determined by the pre-capillary reaction. The Michaelis-Menten constants are also summarized in Table 1. The $K_{m,ATP}$ and $K_{m,ADP}$ determined by pCE/DFA are in good agreement with literature values [41-44].

Fig. 6

Comparison of the Michaelis-Menten Constants

It is noticed from Table 1 that the Michaelis-Menten constants determined by pCE/DFA are somewhat smaller than the ones determined by pre-capillary reactions. Michaelis-Menten constant is defined as dissociation constant of the enzyme-substrate complex, ES, as is written in Eq. (3) with an equilibrium constant (4).

$$\mathrm{E} + \mathrm{S} \underset{K_m}{\rightleftharpoons} \mathrm{ES} \longrightarrow \mathrm{E} + \mathrm{P} \qquad (3)$$

$$K_m = \frac{[\mathrm{E}][\mathrm{S}]}{[\mathrm{ES}]} \qquad (4)$$

where E, S, and P are an enzyme, a substrate, and a product. The product P is continuously resolved from the reaction field by the pCE/DFA, and the formation of ES would be promoted. In other words, the inhibition with the product is

eliminated in pCE/DFA. Traditional analysis methods are generally operated in a homogeneous batch solution, and product inhibitions have been avoided and corrected by measuring the initial reaction rate before the product increase. The traditional methods, however, do not reflect the steady-state of the forward or the backward reaction, and quasi steady-states have been used instead. In the pCE/DFA, the plateau signal was detected with one of the products, ADP or ATP, and the plateau signals of the product were flat, suggesting the kinetic reaction to be in a steady-state. Therefore, it is demonstrated that the pCE/DFA is a promising method to measure the kinetic reaction in a steady-state and to determine the Michaelis-Menten constant without any inhibition by the product.

Conclusions

In this study, the transphosphorylation between creatine and creatine phosphate with CK was analyzed by pCE/DFA. Both reaction products of ADP or ATP in the forward or the backward reactions were detected as a plateau signal under the steady-state. Michaelis-Menten constants of the forward and the backward reactions were successfully determined without any interference from the product or the reverse reaction. The K_m values determined by the pCE/DFA are smaller than the ones determined by the pre-capillary reaction. The result also supports the exclusion of the product inhibition. The exclusion characteristics of the reverse reaction by the proposed pCE/DFA would be beneficial for the practical analysis of the enzyme assays. For the enzymatic reaction in microenvironments such as cell interior, the reaction product would promptly be removed and the inhibition from the product would be little. Therefore, the proposed pCE/DFA would be practical to reproduce the dynamic reactions in cells or in body fluids.

Declarations:

Conflicts of interest/Competing interests: The authors declare that they have no conflict of interest.

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Table 1 Michaelis-Menten constants determined by pCE/DFA or pre-capillary reaction

	Michaelis-Menten constant / mmol L^{-1}		
Method	$K_{m,ATP}$	$K_{m,ADP}$	Reference
Pre-capillary reaction *	0.58±0.03	1.91±0.16	This study
pCE/DFA *	0.39±0.03	1.08±0.10	This study
Circular dichroism	0.40		[41]
Circular dichroism	0.45		[42]
Ion exchange chromatography		1.18	[43]
Circular dichroism		1.0-1.5	[44]

* Error: standard error.

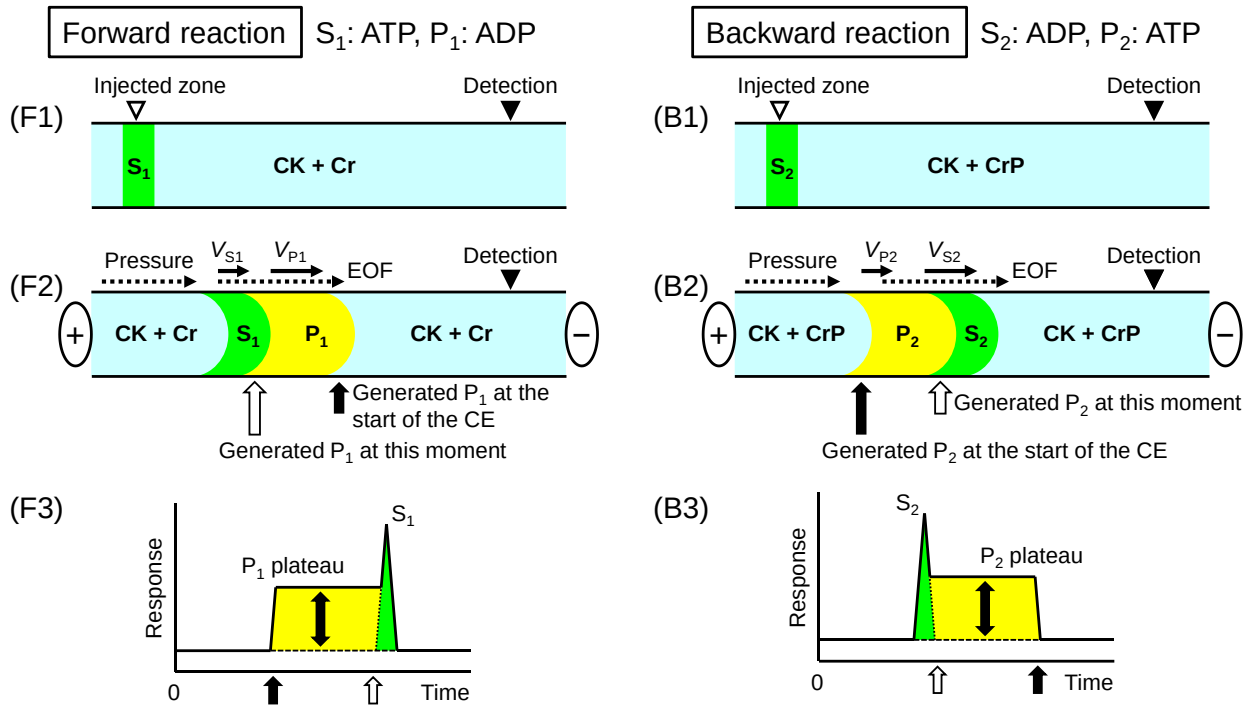

Fig. 1 Schematic illustration of the forward and the backward reactions with creatine kinase in pCE/DFA and expected electropherograms. Details are written in the text.

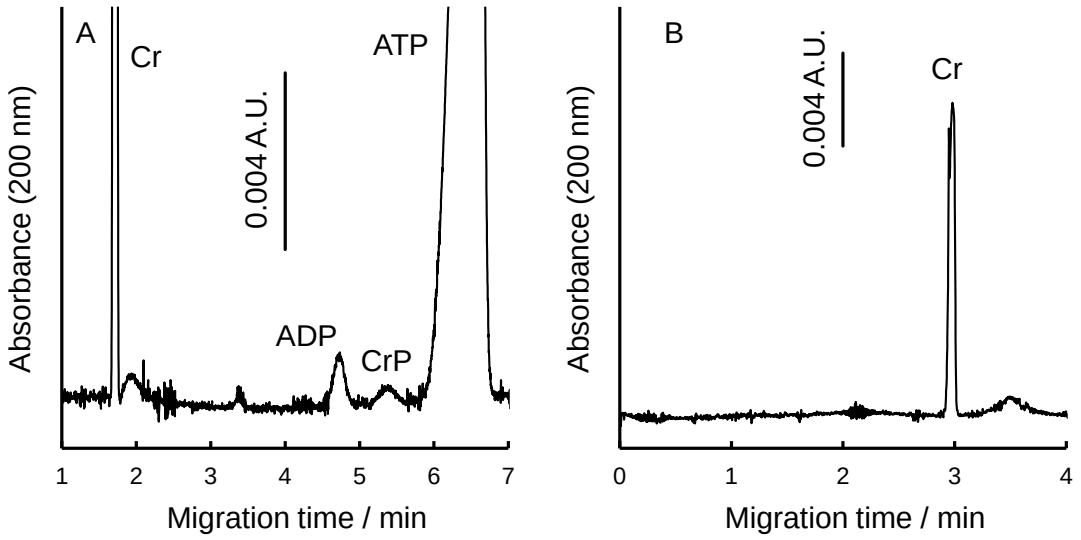

Fig. 2 Typical electropherograms for the forward (A) and the backward (B) reactions with CK by the pre-capillary reaction. A: Formation of CrP from Cr. Initial concentrations of Cr and ATP are 8.0 mmol L^{-1} and 1.0 mmol L^{-1}, respectively. Pressure assist was used for the detections of all the substrates and the products. The pCE conditions are written in the text. B: Formation of Cr from CrP. Initial concentrations of CrP and ADP are 2.0 mmol L^{-1} and 1.0 mmol L^{-1}, respectively. Pressure assist was not used for the single detection of the product of Cr. The CE conditions are written in the text.

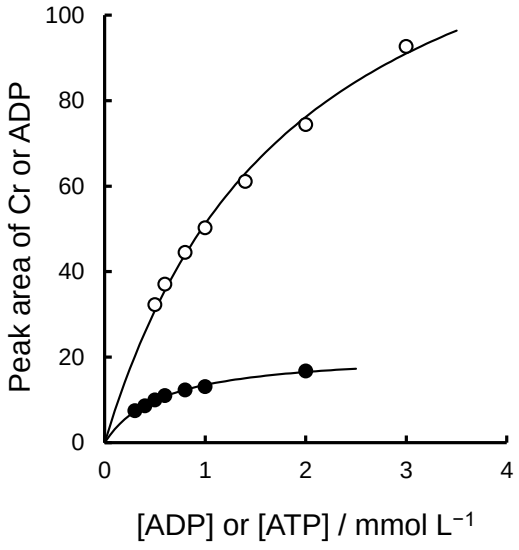

Fig. 3 Non-linear Least-squares analyses for the forward reaction (●) monitored with ADP and for the backward reaction (○) monitored with Cr by the pre-capillary reaction.

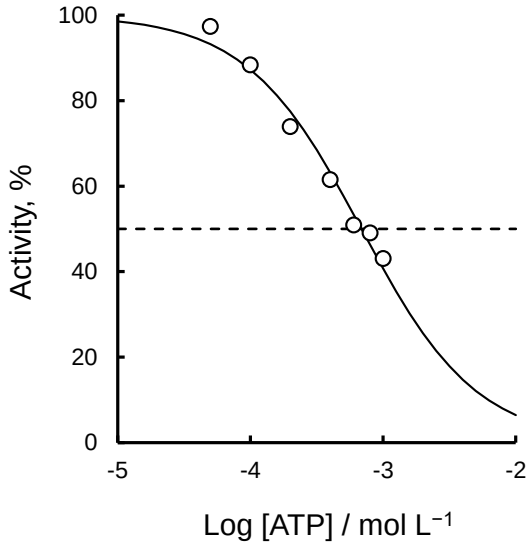

Fig. 4 Activity decrease of the backward reaction with increasing concentrations of ATP. The curve is drawn with an equation, activity = $100/(1+[ATP]/IC_{50})$.

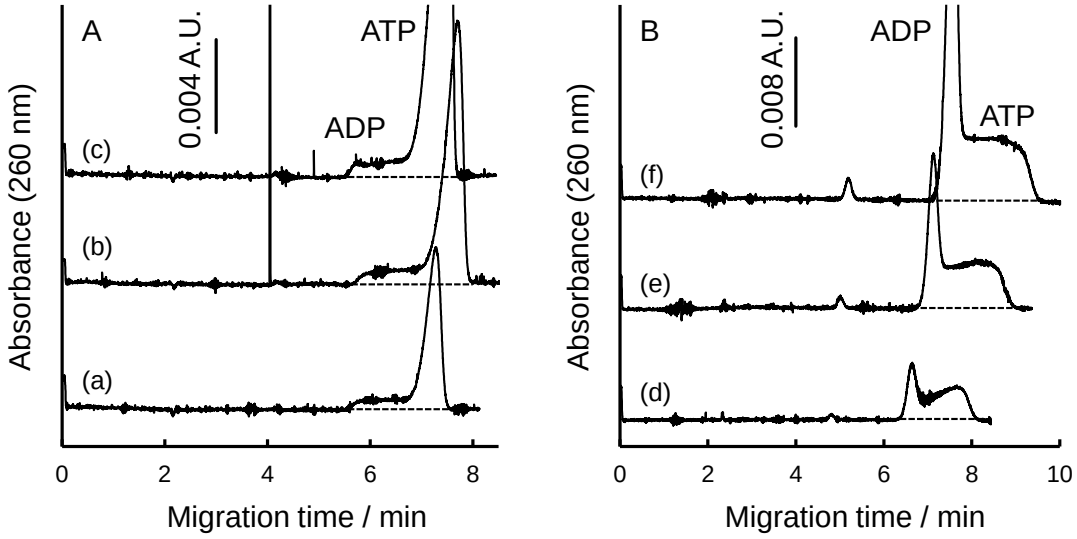

Fig. 5 Typical electropherograms of the forward (A) and backward (B) reactions by the pCE/DFA. A, Concentration of ATP: (a) 0.3 mM, (b) 0.5 mM, (c) 1.0 mM. B, Concentration of ADP: (d) 0.5 mM, (e) 1.0 mM, (f) 2.0 mM. pCE conditions are written in the text.

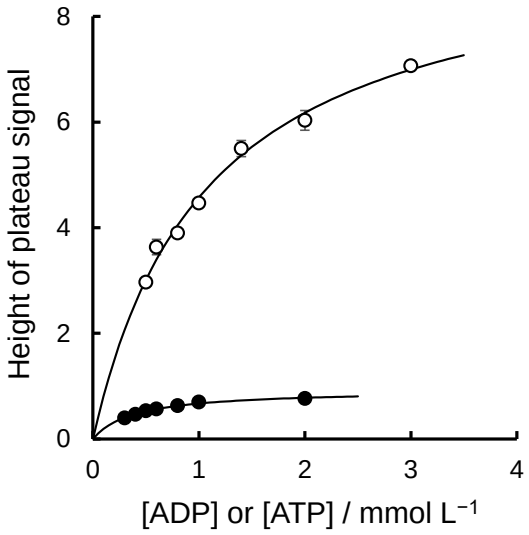

Fig. 6 Non-linear Least-squares analyses for the forward reaction (●) monitored with ADP and for the backward reaction (○) monitored with ATP by the pCE/DFA. The error bars represent the standard deviations.

Graphical abstract